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# Development of Alginate-Based *Bacillus subtilis* Biopellets for Tomato Bacterial Wilt Control

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**Abstract**

Tomato bacterial wilt, caused by *Ralstonia solanacearum*, poses a significant challenge to crop production. This study screened 101 bacterial isolates from soil samples collected in Maha Sarakham Province, Thailand, for their antagonistic activity against *R. solanacearum*. The selection process involved initial screening based on visual inhibition zones using the paper disc diffusion method, followed by quantitative assessment of bacterial growth inhibition. Among the isolates, SK3-24 exhibited the highest inhibitory effect, forming an inhibition zone of 40.00 ± 17.32 mm, as measured using the standard zone of inhibition method, followed by SK3-20 (18.17 ± 1.50 mm). Morphological, biochemical, and molecular analyses (16S rRNA gene sequencing) identified SK3-24 as *Bacillus subtilis*. To enhance its application, SK3-24 was formulated into alginate-based biopellets, which maintained high bacterial viability ($1.20 \times 10^8$ CFU/g) after nine months of storage at 4 °C. Greenhouse trials demonstrated that these biopellets effectively suppressed disease incidence and severity in tomato seedlings and seeds. At 21 days post-inoculation, treated plants showed no disease symptoms and exhibited enhanced growth, with plant height (16.14 ± 1.32 cm) and leaf number (19.40 ± 1.34) comparable to healthy controls. These findings underscore the potential of SK3-24 biopellets as a sustainable and effective biocontrol strategy for managing tomato bacterial wilt while promoting plant health.

**Keywords:** Alginate encapsulation, *Bacillus subtilis*, Biocontrol, Biopellets, Seed treatment, Tomato bacterial wilt

## Introduction

Tomato (*Solanum lycopersicum* Mill.) is one of the most important horticultural crops worldwide, valued for its economic and nutritional significance [1]. Tomatoes are rich in lycopene, vitamins, carotenoids, dietary fiber, essential minerals (such as copper, potassium, and manganese), and flavonoids, making them a vital food source [2]. They offer several health benefits, including reducing the risk of hypertension, cardiovascular disease, and other chronic conditions [3]. Tomatoes are consumed fresh in salads and cooked dishes and serve as a key ingredient in processed products such as sauces, soups, and ketchup. For processed food production, desirable traits include vibrant color, aroma, high soluble solids, and low pH [4]. Despite its nutritional and economic importance, tomato production faces challenges from bacterial wilt, caused by the soil- borne pathogen *Ralstonia solanacearum*. This devastating pathogen is particularly problematic in tropical and subtropical regions, causing significant yield losses in tomatoes and other crops [5]. The pathogen infects plants at all growth stages, entering through root wounds or natural openings. It colonizes the plant's water-conducting system, leading to tissue browning, blockage of water transport, and eventual plant wilting and death [6]. Symptoms typically begin with wilting of the plant's upper parts

during the day, progresing to the permanent wilting of the entire plant. Current management of *R. Solanacearum* is challenging due to its wide host range, genetic diversity, and ability to persist in soil and water for extended periods [7]. Traditional control methods, such as chemical pesticides and crop rotation, are limited in their effectiveness. Chemical pesticides not only have a high environmental impact but also pose risks to human health through contamination of food and water sources. Crop rotation may offer some relief but is not always feasible or effective, particularly in areas with heavy pathogen pressure [8]. Additionally, the emergence of pesticide- resistant strains of *R. Solanacearum* further complicates disease management, creating a critical need for alternative, sustainable control strategies.

Biological control using antagonistic bacteria offers an eco- friendly approach to managing bacterial wilt. Among the potential biocontrol agents, *Bacillus subtilis* stands out for its ability to produce antimicrobial compounds, colonize plant roots, and form endospores that enable long-term survival under adverse conditions [9]. Additionally, bioformulations, such as alginate biopellets, enhance the stability and efficacy of biocontrol agents, providing a scalable solution for field applications [10]. Other antagonistic bacteria, including *Pseudomonas* spp. And *Pantoea agglomerans*, have also demonstrated significant inhibitory effects against *R. solanacearum*, reducing disease severity and increasing crop yields [6].

This study aims to fill the knowledge gap regarding effective biological control agents against *R. Solanacearum* by screening and identifying antagonistic bacterial strains. Through 16S rDNA sequencing, this research will identify *B. Subtilis* and other potential biocontrol agents with strong inhibitory effects against the pathogen. Furthermore, this study will develop a bioformulation in the form of alginate- based biopellets to improve the stability, viability, and effectiveness of these biocontrol agents under field conditions. The goal is to provide an environmentally friendly, scalable alternative to chemical pesticides for the sustainable management of tomato bacterial wilt, addressing the limitations of traditional control methods while promoting enhanced tomato production.

## Materials and methods

### Tomato wilt disease pathogen

The causative agent of tomato wilt, *Ralstonia solanacearum* was obtained from the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University. Cultures of *R. Solanacearum* preserved in 20% glycerol were inoculated on Triphenyl tetrazolium chloride (TZC) medium to obtain pure cultures. The purified cultures were subsequently stored on nutrient agar (NA) slants for further experimentation.

### Isolation of antagonistic bacteria from soil

Soil samples were collected from two locations in Maha Sarakham Province: Bo Yai Village, Borabue District (16° 2'18" N, 103°7'9"E) and Don Na Village, Kantharawichai District (16° 19'22"N, 103° 17'48"E). Two soil samples were randomly selected from each area, with 3 sampling points per area. For each sample, soil was collected from 3 points around a tomato plant at a depth of 15 - 30 cm. Each 10 g soil sample was air-dried and thoroughly mixed before subsampling. A 10 g portion of soil was added to 90 mL of sterile distilled water, shaken at 150 rpm for 30 min, and serially diluted ($10^{-1}$ to $10^{-6}$). Dilutions of $10^{-4}$, $10^{-5}$, and $10^{-6}$ were plated on nutrient agar (NA) in triplicate and incubated at 28 ± 2 °C for 24 - 48 h. Isolated colonies were purified for further testing.

### Antagonistic activity screening

Initial screening: The antagonistic efficiency of bacterial isolates was assessed using the paper disc diffusion method. *R. solanacearum* was cultured in nutrient broth (NB) for 24 h under shaking conditions (150 rpm). The bacterial culture was adjusted to an optical density (OD) of 0.2 at 600 nm (approximately $1 \times 10^8$ CFU/mL). *R. Solanacearum* was swabbed onto NA plates. Paper discs loaded with 20 µL of antagonistic bacterial suspension were placed on the plates, which were incubated at 28 ± 2 °C for 48 h. Zones of inhibition were measured in millimeters [11].

Secondary screening: Effective isolates from the initial test were further evaluated using a single paper disc method. A single disc containing 20 µL of each bacterial suspension was placed at the center of an agar plate inoculated with *R. solanacearum*. Plates were incubated under the same conditions, and inhibition zones were measured in triplicate.

**Efficiency test of antagonistic bacteria in controlling tomato wilt pathogen on tomato seeds**

Preparation of bacterial cultures: Antagonistic bacteria and *R. Solanacearum* were grown in NB for 24 h. The cultures were adjusted to an OD of 0.2 at 600 nm (approximately $1 \times 10^8$ CFU/mL).

Preparation of test seeds: Tomato seeds (*Lycopersicon esculentum* Mill., Sida variety) were surface sterilized by soaking in a 10 % Clorox solution for 3 min, followed by 2 rinses with sterile distilled water to remove any residual bleach. The sterilized seeds were then soaked in bacterial suspensions (*B. subtilis* at a concentration of $1 \times 10^8$ CFU/mL) for 30 min, allowing for effective bacterial adhesion. After soaking, the seeds were air- dried under sterile conditions before use in the experiment.

Experimental setup: Sterile germination test papers were placed on sterile plates, and 5 mL of *R. solanacearum* suspension was added. Five treated seeds were placed on each plate. Four treatments were performed with three replicates: (1) Sterile distilled water + Seeds soaked in sterile water (Control), (2) *R. Solanacearum* + Seeds soaked in sterile water, (3) *R. Solanacearum* + Seeds soaked in 100 ppm streptomycin and (4) *R. Solanacearum* + Seeds soaked in each antagonistic bacterial isolate.

Observation and data collection: The number of diseased plants was recorded after 14 days. Disease incidence (%) was calculated as: Disease incidence (%) = (Number of diseased tomato plants / Total number of tomato plants) × 100.

**Efficiency test of antagonistic bacteria in controlling tomato wilt disease on tomato seedlings**

Preparation of *R. solanacearum* and antagonistic bacteria: Both *R. Solanacearum* and the most effective antagonistic bacterial isolate were prepared as described above.

Preparation of test plants: Tomato seeds were surface sterilized and sown in sterilized peat moss. One-week-old and two-week-old seedlings were transplanted into pots containing sterilized peat moss (100 g/pot).

Experimental setup: Pots were inoculated with 10 mL of *R. Solanacearum* suspension. After 24 h, antagonistic bacteria (10 mL) were applied. The treatments included: (1) Soil without *R. solanacearum*, (2) Soil with *R. solanacearum*, (3) Soil with *R. Solanacearum* + 100 ppm streptomycin, (4) Soil with *R. Solanacearum* + *Bacillus* bio-product No. 1, (5) Soil with *R. Solanacearum* + Each antagonistic isolate and (6) Soil with antagonistic isolate only (no *R. solanacearum*).

Data collection: The percentage of disease incidence was recorded 14 days after inoculation.

**Identification of effective antagonistic bacteria**

Morphological and biochemical classification: Colony morphology and Gram staining were used for initial identification. Biochemical tests included catalase, citrate utilization, indole, motility, MR (Methyl Red), oxidase, starch hydrolysis, urease, VP (Voges Proskauer) and gelatin digestion.

Molecular identification: The 16S rRNA genes of effective isolates were sequenced (Macrogen, Korea) using primers 27F and 1492R. The sequences were compared with GenBank data, and phylogenetic relationships were analyzed.

**Preparation of effective antagonistic bacteria bioformulation**

Encapsulation and bioformulation: Bacterial suspensions were prepared at a concentration of $1 \times 10^9$ CFU/mL. The polymer solution was prepared by mixing 1 % (w/v) skim milk, 1.5 % (w/v) sodium alginate, 10 % (w/v) rice bran, and 0.5 % (w/v) glucose into sterile distilled water. Specifically, 100 g of rice bran, 15 g of sodium alginate, 10 g of skim milk, and 5 g of glucose were dissolved in 900 mL of sterile distilled water. Then, 100 mL of the bacterial suspension was added to the polymer solution under gentle stirring to ensure uniform distribution. The resulting mixture was dropped using a sterile syringe into a 0.25 M $CaCl_2$ solution to form spherical biopellets by ionic gelation. The biopellets were left in the $CaCl_2$ solution for 30 min to complete gelation, then collected by filtration and washed twice with sterile distilled water. The pellets were dried at 55 °C under sterile conditions for 24 h and subsequently stored at 4 °C until further use.

Characterization of biopellets: Size, shape, and surface properties of biopellets were analyzed using a stereo microscope and SEM. Survival rates of bacteria in biopellets were assessed at 0, 60, and 90 days post-storage by the total plate count method.

**Efficacy test of biopellets in controlling *Ralstonia solanacearum***

Preparation of soil and test plants: Soil was prepared by mixing loose soil, compost, and coconut husks (1: 1: 1 ratio). Tomato seedlings (14 days olds) were transplanted into soil inoculated with *R. Solanacearum* (200 mL suspension per pot).

Experimental Treatments: (1) Positive control: No pathogen inoculation, (2) Negative control: *R. Solanacearum* only, (3) Biopellets: *R. Solanacearum* + biopellets at planting hole bottom and (4) Chemical: *R. Solanacearum* + 100 ppm Streptomycin at planting hole bottom.

Data collection: Plant growth was monitored weekly (height, leaf count). Disease incidence and severity were assessed weekly. The degree of wilt in each seedling was evaluated individually using a 0 - 4 scale: A score of 0 indicated no symptoms (no wilting); a score of 1 indicated wilting of 25% of the leaves; a score of 2 indicated wilting of 50% of the leaves; a score of 3 indicated wilting of 75% of the leaves; and a score of 4 indicated complete wilting or plant death. The disease severity index (DSI) was calculated to evaluate the bacterial wilt (BW) reaction in each tomato accession, using the following formula: DSI = $100 \times \sum$ (frequency × rating score) / [(total number of observations) × (maximum disease score)] [7].

Statistical analysis: Data were analyzed using ANOVA with mean separation by least significant difference (LSD) test at $p < 0.05$.

**Results and discussion**

**Selection of antagonistic bacteria from soil**

The total of 101 isolates collected from Bo Yai Village and Don Na Village represents a diverse microbial population that may harbor beneficial traits for plant disease suppression. This aligns with previous studies indicating that soil microbiota play a crucial role in natural disease resistance by outcompeting pathogens through resource competition, antibiosis, and induction of plant defense mechanisms [12]. The difference in the number of isolates obtained from the 2 locations suggests variations in soil microbial diversity, which could be influenced by factors such as soil type, organic matter content, and land use history. Bo Yai Village yielded 37 isolates, whereas Don Na Village provided 64 isolates, potentially indicating more favorable conditions for bacterial proliferation in the latter site. Similar findings were reported by Jayaraman *et al*. [13], who demonstrated that soil properties significantly affect the abundance and composition of antagonistic bacteria.

**Antagonistic activity screening**

In the initial screening, all 101 bacterial isolates were tested using the paper disc diffusion method to evaluate their ability to inhibit the growth of *R. solanacearum*. The inhibition zones ranged from 6.83 ± 1.53 mm to 40.00 ± 17.32 mm. The isolate SK3-24 demonstrated the strongest activity, with an inhibition zone of 40.00 ± 17.32 mm. Other high-performing isolates included SK3-19 (35.00 ± 0 mm), SK2-8 (32.50 ± 4.33 mm), and SB2-6 (28.67 ± 0.20 mm). Conversely, isolates such as SK1-3 (6.83 ± 1.53 mm), SK2-6 (7.00 ± 0 mm), and SK3-21 (7.33 ± 0.76 mm) exhibited weak inhibition.

Secondary screening, 22 bacterial isolates, along with Streptomycin (positive control), $dH_2O$ (negative control), and *Bacillus* No. 1 (a commercial bioproduct strain), were subjected to secondary screening. Streptomycin displayed the strongest inhibition, with a zone of 32.17 ± 2.02 mm (**Table 1**). Among the isolates, SK3-24 showed the highest efficacy (22.83 ± 2.47 mm), followed by SK3-20 (18.17 ± 1.50 mm) and *Bacillus* No. 1 (19.00 ± 1.73 mm).

The results demonstrate that SK3-24 and SK3-20 are promising candidates for biocontrol applications against *R. solanacearum*. The strong inhibition zones observed in the screening assays suggest the production of potent antimicrobial compounds. Previous studies have reported similar findings, where *Bacillus subtilis* and *Pseudomonas fluorescens* exhibited strong antagonistic effects against bacterial pathogens [14,15]. Variability in inhibition zones among isolates may be attributed to differences in secondary metabolite production. High-performing isolates like SK3-24 may produce broad-spectrum antimicrobial compounds, whereas weak-performing isolates such as SK2-8 might have limited antimicrobial activity. In particular, *B. Subtilis* SK3-24 likely exerts its inhibitory effects through the production of bioactive secondary metabolites such as lipopeptides (e.g., surfactin, iturin, and fengycin). These lipopeptides can disrupt the integrity of pathogen cell membranes by inserting

themselves into the lipid bilayer, leading to pore formation, leakage of intracellular contents, and subsequent cell lysis. At the molecular level, *B. Subtilis* SK3-24 may also produce antimicrobial peptides, such as bacteriocins, which can inhibit the growth of *R. Solanacearum* by interfering with key metabolic pathways or by directly binding to receptors on the bacterial cell surface. Additionally, *B. Subtilis* SK3-24 is known to secrete hydrolytic enzymes, including chitinases and cellulases, which can degrade the cell walls of pathogens, further contributing to the suppression of *R. solanacearum*. These molecular mechanisms are consistent with the observed strong antagonistic activity of SK3-24. The production of these substances likely interferes with various vital processes in *R. solanacearum*, such as cell wall synthesis and membrane integrity, which impairs pathogen survival. Similar trends have been observed in studies evaluating soil bacteria for disease suppression [16,17].

**Table 1** Efficacy testing of antagonistic bacteria for controlling tomato wilt disease by paper disc diffusion method.

| Isolates | Inhibition zone diameter (mm) |
|---|---|
| SB1-3 | $9.50 \pm 0.00^{efg}$ |
| SB2-2 | $11.67 \pm 0.50^{ef}$ |
| SB2-6 | $12.67 \pm 0.76^{def}$ |
| SB2-7 | $11.33 \pm 0.76^{efg}$ |
| SB2-12 | $8.50 \pm 1.04^{fg}$ |
| SB3-1 | $10.00 \pm 0^{efg}$ |
| SB3-6 | $13.00 \pm 2.80^{def}$ |
| SK1-5 | $10.83 \pm 0.87^{efg}$ |
| SK1-6 | $10.83 \pm 1.04^{efg}$ |
| SK1-16 | $11.67 \pm 1.04^{ef}$ |
| SK1-22 | $11.33 \pm 1.26^{efg}$ |
| SK2-7 | $12.50 \pm 0.29^{def}$ |
| SK2-8 | $5.67 \pm 0^{gh}$ |
| SK3-3 | $12.00 \pm 0.29^{ef}$ |
| SK3-6 | $10.33 \pm 0.29^{efg}$ |
| SK3-13 | $11.33 \pm 0.50^{efg}$ |
| SK3-14 | $11.33 \pm 2.52^{efg}$ |
| SK3-17 | $11.33 \pm 0.29^{efg}$ |
| SK3-19 | $14.50 \pm 0.50^{cde}$ |
| SK3-20 | $18.17 \pm 1.50^{bcd}$ |
| SK3-22 | $12.67 \pm 0.68^{def}$ |
| SK3-24 | $22.83 \pm 2.47^{b}$ |
| $dH_2O$ | $0.000 \pm 0.00^{h}$ |
| Streptomycin (100 ppm) | $32.17 \pm 2.02^{a}$ |
| *Bacillus* No. 1 | $19.00 \pm 1.73^{bc}$ |

Values are expressed as mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$).

**Efficacy test of biopellets in controlling *Ralstonia solanacearum***

The efficacy of antagonistic bacteria in controlling *R. Solanacearum* on test seeds was also evaluated. The untreated control group exhibited the highest disease incidence (73.33 ± 11.55%), significantly greater than all other treatments (**Table 2**). Co-inoculation with SK3-24 reduced the disease incidence to 43.33 ± 11.55%, while Streptomycin (100 ppm) achieved the lowest disease incidence (40.00 ± 10.00%)

**Table 2** Efficacy of antagonistic bacteria for controlling tomato wilt disease on test seeds, 14 days after inoculation.

| Treatment | Disease Incidence (%) |
|---|---|
| *R. solanacearum* + SK3-20 | 56.67 ± 11.55 ab |
| *R. solanacearum* + SK3-24 | 43.33 ± 11.55 b |
| *R. solanacearum* Streptomycin (100 ppm) | 40.00 ± 10.00 b |
| *R. solanacearum* | 73.33 ± 11.55 a |
| $dH_2O$ | 0.00 ± 0.00 c |

Values are expressed as mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$).

The efficacy of antagonistic bacteria in controlling *R. Solanacearum* was assessed 14 days after inoculation. At 7 days old seedlings, the untreated control group showed the highest disease incidence (100.00 ± 0.00%). Co-inoculation with SK3-24, SK3-20, or Streptomycin resulted in reduced disease incidences of 86.67 ± 5.77%, 96.67 ± 5.77%, and 96.67 ± 5.77%, respectively (**Table 3**). At 14 days old seedlings, the untreated control maintained a 100.00 ± 0.00% disease incidence. SK3-24 exhibited the lowest disease incidence (13.33 ± 5.77%), followed by Streptomycin (10.00 ± 0.00%).

**Table 3** Efficacy of antagonistic bacteria for controlling tomato wilt pathogen on -7 day and -14 day-old seedlings, 14 days after inoculation.

| Treatment | Disease Incidence (%) | |
|---|---|---|
| | 7 days olds seedling | 14 days olds seedling |
| *R. solanacearum* + SK3-20 | 96.67 ± 5.77 ab | 33.33 ± 5.77 b |
| *R. solanacearum* + SK3-24 | 86.67 ± 5.77 ab | 13.33 ± 5.77 d |
| SK3-20 | 0.00 ± 0.00 b | 0.00 ± 0.00 e |
| SK3-24 | 0.00 ± 0.00 b | 0.00 ± 0.00 e |
| *R. solanacearum* + Streptomycin (100 ppm) | 96.67 ± 5.77 ab | 10.00 ± 0.00 d |
| *R. solanacearum* + *Bacillus* No. 1 | 80.00 ± 20.00 ab | 23.33 ± 5.77 c |
| *R. solanacearum* | 100.00 ± 0.00 a | 100.00 ± 0.00 a |
| $dH_2O$ | 0.00 ± 0.00 b | 0.00 ± 0.00 e |

Values are expressed as mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$).

The efficacy of SK3-24 in seed and seedling assays further confirms its biocontrol potential. Its performance was comparable to Streptomycin, aligning with previous findings that *Bacillus* species can serve as effective biocontrol agents [15]. The mechanism of action likely involves competition, production of antimicrobial metabolites, and induction of systemic resistance in plants. *B. Subtilis* competes with plant pathogens for nutrients and space, preventing their colonization of the plant's roots or surface. It also produces antimicrobial compounds such as bacitracin, surfactin, and iturin, which inhibit pathogen growth by disrupting their cell walls, membranes, and metabolic processes. Additionally, *B. Subtilis* triggers plant defense pathways, enhancing the plant's resistance to pathogens by activating genes that produce defense proteins and strengthen cell walls. These combined mechanisms provide effective protection against plant diseases, offering a sustainable alternative to chemical pesticides [18]. These findings support the potential of SK3-24 as an eco-friendly alternative to chemical pesticides. Future research should focus on characterizing the active compounds produced by SK3-24 and SK3-20, assessing their efficacy in greenhouse and field trials, and exploring their formulation for commercial use. Additionally, understanding the genetic basis of antagonism in these isolates could

provide insights for optimizing biocontrol strategies [14].

**Identification of effective antagonistic bacteria**

The morphological characteristics of the antagonistic bacterial isolate SK3-24 revealed a round to irregular, opaque white colony with serrated edges. Microscopic analysis showed that SK3-24 is a rod-shaped, Gram-positive bacterium that forms endospores.

Biochemical tests further supported the classification of SK3-24. The isolate exhibited positive reactions for catalase, oxidase, and starch hydrolysis, which are typical characteristics of *Bacillus subtilis*. However, SK3-24 differed from *B. subtilis* in its ability to utilize citrate (**Table 4**).

**Table 4** Biochemical properties of antagonistic bacterial isolate SK3-24.

| Test | SK3-24 | *Bacillus subtilis*[1/] |
|---|---|---|
| Shape | Rod | Rod |
| Spore | Positive | Positive |
| Gram stain | Positive | Positive |
| Catalase | + | + |
| Citrate utilization | − | + |
| Oxidase | + | + |
| Indole | − | − |
| Motility | + | + |
| MR (Methyl Red) | − | − |
| Starch hydrolysis | + | + |
| Urease | − | − |
| VP (Voges Proskauer) | + | + |

Bergey's Manual of Systematic Bacteriology (Boone *et al.*, 2001) [19].

The molecular identity of SK3-24 was determined using the 16S rRNA gene sequence. A comparative analysis with sequences in the GenBank database confirmed that SK3-24 belongs to the genus *Bacillus* and shares a high degree of similarity with *B. subtilis*. The phylogenetic tree (**Figure 1**) further supports the classification of SK3-24 as *B. subtilis*. The close clustering of SK3-24 with other *Bacillus subtilis* strains highlights its genetic relatedness within this group.

The combined morphological, biochemical, and molecular evidence confirms that SK3-24 is a strain of *B. subtilis*. Morphological and biochemical analyses are foundational for bacterial identification; however, the addition of molecular techniques, such as 16S rRNA sequencing, provides a more robust classification by overcoming the limitations of phenotypic variability [20]. The biochemical profile of SK3-24 aligns with that of *B. Subtilis* described in Bergey's Manual, though its negative result for citrate utilization distinguishes it from standard strains. This variability highlights the potential for strain-specific metabolic adaptations, which could influence its application as a biocontrol agent. The identification of SK3-24 as *B. Subtilis* aligns with previous studies reporting the effectiveness of this species in biocontrol due to its ability to produce secondary metabolites, including lipopeptides and enzymes, that suppress plant pathogens [21, 22]. Further investigation into the functional metabolites of SK3-24 is warranted to optimize its use in managing *R. Solanacearum* and other agricultural pathogens.

**Preparation of effective antagonistic bacterial bioformulation**

***Characterization of biopellets***

Random sampling and examination of biopellets under a stereo microscope revealed distinct physical characteristics. The alginate- based biopellets were

primarily round to oval in shape, with particle dimensions ranging from approximately 2 - 3.5 mm in width and 2.5 - 3.5 mm in length. The pellets exhibited a brown-gray coloration and an irregular surface texture. Further structural analysis was conducted using a scanning electron microscope (SEM) (Jeol 6480LV, Japan). SEM images at various magnifications 50x, 200x, 500x, and 2,000x revealed a rough and uneven surface morphology (**Figure 2**). No bacterial cells were observed on the external surfaces, indicating that the bacteria were successfully encapsulated within the alginate matrix, thereby preventing external exposure.

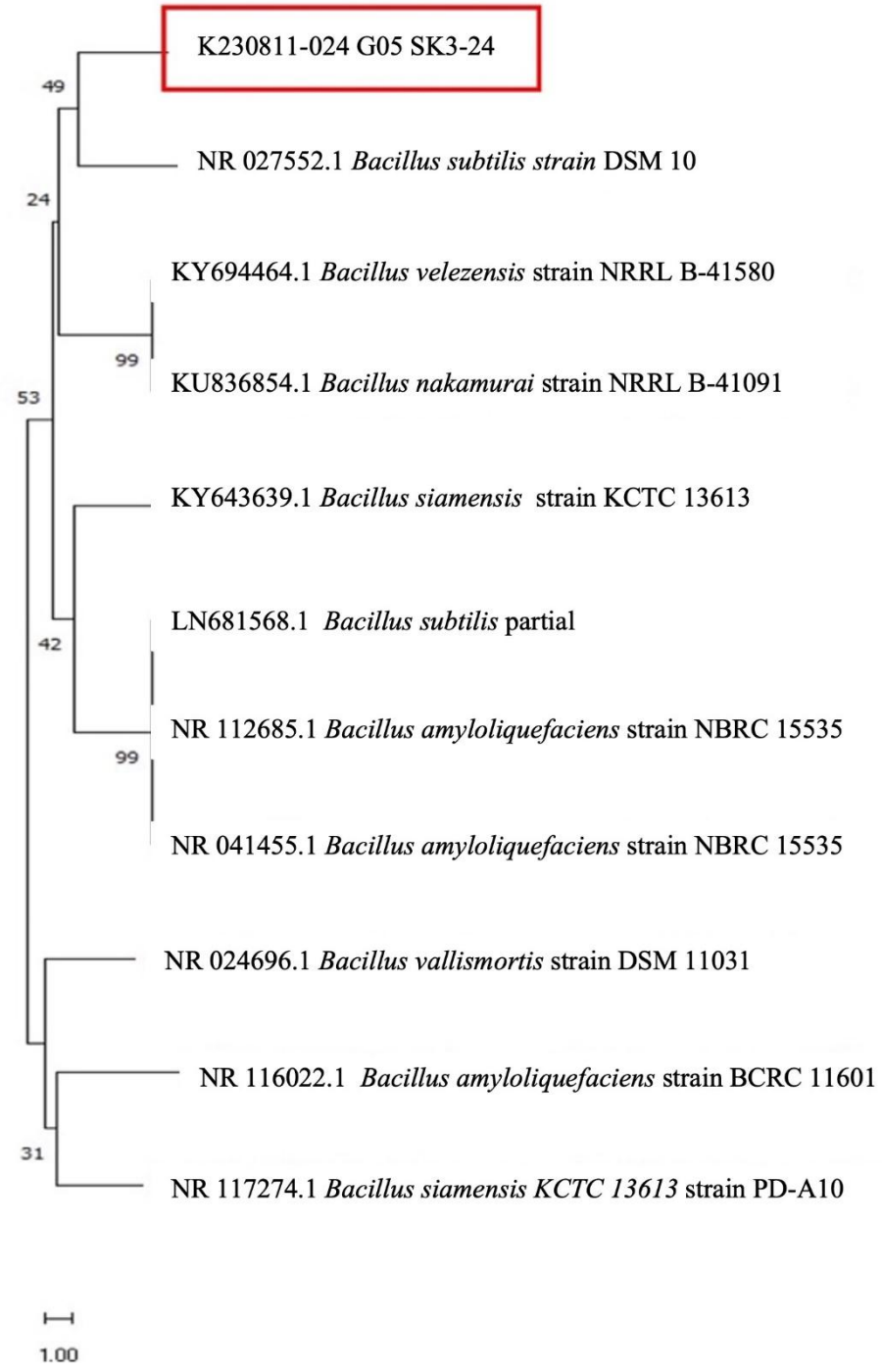


**Figure 1** Phylogenetic tree of the antagonistic bacterial isolate SK3-24.

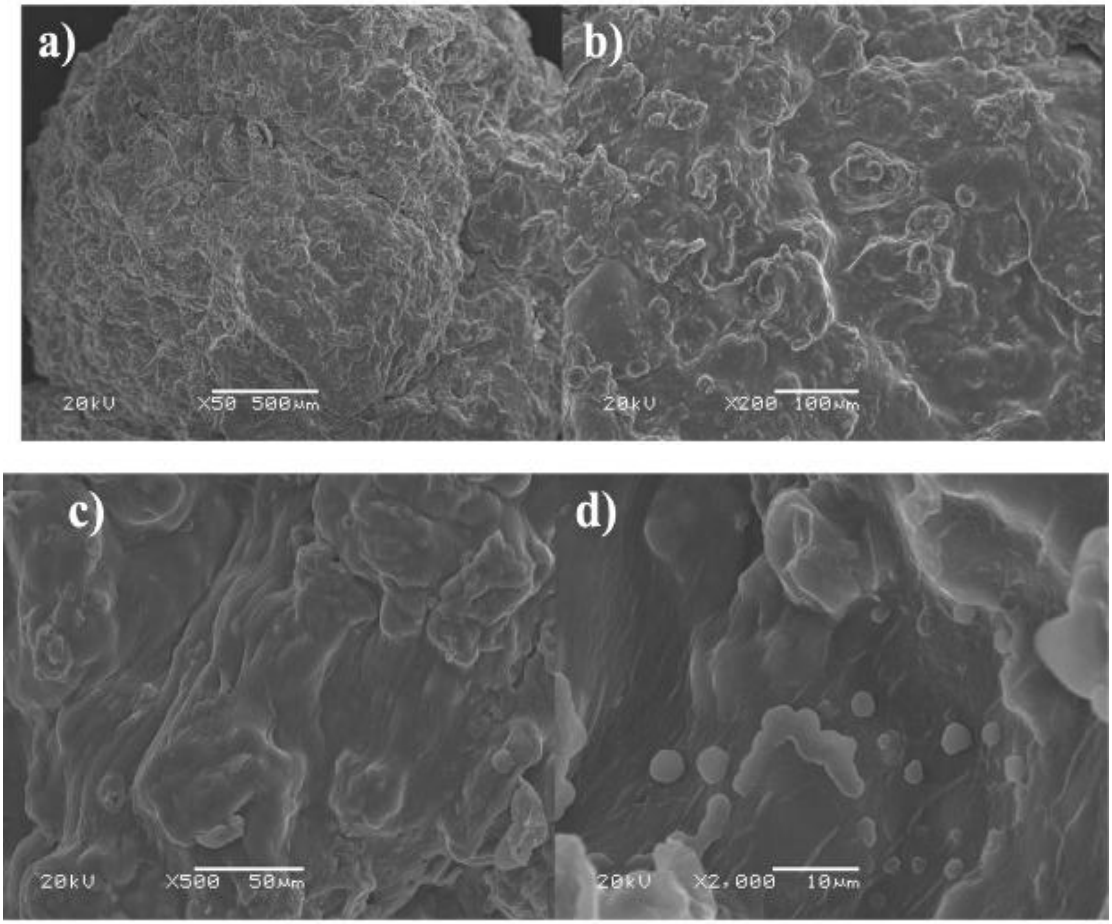


**Figure 2** The characteristics of the alginate-based biopellets were examined using a scanning electron microscope (SEM). (a) magnification 50x, (b) magnification 200x, (c) magnification 500x, (d) magnification 2,000x.

The observed physical characteristics of the alginate- based biopellets align with previous studies demonstrating that alginate encapsulation produces biopellets with non-uniform shapes and surfaces, which can influence their dissolution and microbial release properties [23]. The irregular surface texture may affect hydration and controlled release properties, impacting the efficacy of bioformulations in agricultural applications. Encapsulation within alginate provides several advantages for bacterial bioformulations. It enhances bacterial stability by shielding cells from environmental stressors such as desiccation, ultraviolet light, and oxidative damage, thereby improving their shelf life and field performance [24]. Studies have shown that alginate- encapsulated bacteria maintain higher viability over extended storage periods compared to free bacterial cells, making them an effective delivery method for microbial inoculants [25]. Furthermore, alginate matrices allow for gradual bacterial release in soil environments, improving colonization and persistence of beneficial microbes [26]. Importantly, alginate is a natural, biodegradable polymer widely recognized as safe for the environment and non-target organisms. The use of alginate- based biopellets minimizes the risk of environmental contamination compared to chemical pesticides, as the matrix degrades naturally in soil without leaving harmful residues. Additionally, the encapsulated *B. Subtilis* SK3-24 strain is a beneficial microorganism commonly found in the soil microbiome, and it is generally regarded as safe by regulatory agencies. Therefore, the application of these biopellets is unlikely to pose risks to soil health, non-target plants, animals, or beneficial microorganisms. These findings further support the application of alginate- encapsulated biopellets as a safe and effective means of delivering antagonistic bacteria for sustainable agricultural biocontrol. Future research should focus on optimizing encapsulation conditions to enhance bacterial viability, environmental stability, and field efficacy.

**Survival of effective antagonistic bacteria in biopellets**

The survival test for the encapsulated antagonistic bacteria (SK3-24) revealed an initial bacterial concentration of $1.21 \times 10^9$ CFU/g immediately after biopellet preparation. After storage at 4 °C, the bacterial count decreased to $2.40 \times 10^8$ CFU/g and $1.20 \times 10^8$ CFU/g at 6 and 9 months, respectively. The gradual reduction in bacterial viability over time aligns with studies on encapsulated biocontrol agents, where prolonged storage under refrigeration conditions minimizes metabolic activity but does not eliminate bacterial viability [27]. The bacterial survival rate remains within acceptable thresholds for effective biocontrol applications, as previous research has demonstrated that populations of $10^7$ to $10^8$ CFU/g are sufficient to suppress plant pathogens effectively [28]. The rough surface morphology observed under SEM suggests that the alginate matrix effectively embedded the bacteria internally, a characteristic that enhances protection and controlled release during field application [22]. Storage at 4 °C preserved bacterial viability significantly better than ambient conditions reported in other studies, reaffirming the role of low temperatures in maintaining microbial bioformulations [24]. Future studies could explore additional additives, such as trehalose or skim milk, to further enhance the stability and viability of encapsulated bacteria during storage [27].

**Efficacy of biopellets in controlling tomato wilt pathogens**

The biopellets demonstrated exceptional efficacy in suppressing tomato wilt disease caused by *R. solanacearum*. At both 14 and 21 days after inoculation, the biopellet treatment (*R. Solanacearum* + biopellets) resulted in zero disease incidence and severity (0.00% ± 0.00), statistically comparable to the control group (no pathogens). The streptomycin treatment initially suppressed disease (0.00% ± 0.00 at 14 days) but was less effective over time, with disease incidence and severity reaching 40.00% ± 12.25 and 40.00% ± 15.51, respectively, by 21 days. The untreated group (*R. Solanacearum* + $dH_2O$) exhibited the highest disease incidence and severity, reaching 100.00% ± 0.00 at 21 days after inoculation (**Table 5**).

**Table 5** Efficacy of biopellets in controlling tomato wilt pathogens after 14 and 21 days of inoculation.

| Treatment | Disease incidence (%) | | Disease severity (%) | |
|---|---|---|---|---|
| | 14 d | 21 d | 14 d | 21 d |
| *R. solanacerun* + Biopellets | 0.00 ± 0.00 a | 0.00 ± 0.00 b | 0.00 ± 0.00 a | 0.00 ± 0.00 b |
| *R. solanacerun* + Streptomycin | 0.00 ± 0.00 a | 40.00 ± 12.25 ab | 0.00 ± 0.00 a | 40.00 ± 15.51 ab |
| *R. solanacerun* + $dH_2O$ | 40.00 ± 12.25 a | 100.00 ± 0.00 a | 28.00 ± 9.50 a | 100.00 ± 0.00 a |
| Control (No pathogens) | 0.00 ± 0.00 a | 0.00 ± 0.00 b | 0.00 ± 0.00 a | 0.00 ± 0.00 b |

Values are expressed as mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$).

**Efficacy of biopellets in promoting tomato growth**

The biopellets significantly promoted tomato plant growth, as evidenced by increased plant height and leaf number at 7, 14 and 21 days after inoculation (**Table 6**). At 21 days, plants treated with biopellets showed the highest growth metrics, with plant height (16.14 ± 1.32 cm) and leaf number (19.40 ± 1.34) statistically comparable to the control group. In contrast, the streptomycin-treated plants displayed moderate growth (8.12 ± 3.34 cm height and 9.64 ± 2.02 leaves), while untreated plants infected with *R. solanacearum* showed no measurable growth by 21 days.

**Table 6** Efficacy of biopellets in promoting tomato growth after 7, 14and 21days of inoculation.

| Treatment | Hight (cm) | | | Leaf number | | |
|---|---|---|---|---|---|---|
| | 7 d | 14 d | 21 d | 7 d | 14 d | 21 d |
| *R. solanacerun* + Biopellets | 9.62 ± 0.53 a | 11.84 ± 1.20 a | 16.14 ± 1.32 a | 12.40 ± 1.52 a | 13.80 ± 0.54 a | 19.40 ± 1.34 a |
| *R. solanacerun* + Streptomycin | 8.24 ± 1.31 a | 9.34 ± 0.52 a | 8.12 ± 3.34 bc | 8.60 ± 5.08 ab | 7.84 ± 3.10 ab | 9.64 ± 2.02 ab |
| *R. solanacerun* + $dH_2O$ | 5.56 ± 3.09 a | 2.02 ± 4.53 b | 0.00 ± 0.00 c | 6.64 ± 2.16 b | 1.48 ± 3.09 b | 0.00 ± 0.00 b |
| Control (No pathogens) | 7.34 ± 0.23 a | 9.42 ± 0.62 a | 12.68 ± 0.61 ab | 8.80 ± 1.10 ab | 11.40 ± 0.89 a | 15.40 ± 1.52 a |

Values are expressed as mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$).

The biopellets consistently outperformed the streptomycin treatment and untreated controls, underscoring their robust disease-suppressing capacity. This aligns with findings from previous studies highlighting the effectiveness of biocontrol formulations, particularly those encapsulated in alginate, in mitigating soilborne plant pathogens [24,28]. The inability of streptomycin to maintain efficacy over 21 days may reflect its rapid degradation in soil environments, whereas the sustained activity of the biopellets likely results from the gradual release of the antagonistic bacteria. The observed suppression of *R. Solanacearum* reinforces the potential of biopellets as an alternative to chemical control strategies. The superior growth performance in the biopellet treatment group highlights the dual functionality of this bioformulation in suppressing pathogens and promoting plant growth. This is likely due to the activity of the antagonistic bacteria in reducing pathogen load while simultaneously producing growth-promoting substances such as indole-3-acetic acid (IAA) and solubilizing nutrients in the rhizosphere [29]. The observed growth promotion supports findings from studies that reported improved plant vigor following the application of biocontrol agents encapsulated in alginate matrices [26]. The encapsulation of bacteria in alginate matrices ensures prolonged viability and gradual release, contributing to sustained efficacy over time. The results strongly indicate that biopellets containing antagonistic bacteria are a promising biocontrol strategy for managing tomato wilt caused by *R. solanacearum*. Their ability to suppress disease completely, coupled with their growth-promoting effects, positions them as a sustainable alternative to chemical treatments such as streptomycin. While the biopellets showed no signs of pathogen infection or growth suppression under experimental conditions, field trials are recommended to validate their performance under diverse environmental conditions. Further research should also explore optimization of pellet formulation, including the use of additional stabilizers or nutrient amendments, to enhance bacterial survival and efficacy during storage and application [24]. These findings highlight the potential of biopellets as an eco-friendly approach to managing soilborne diseases while promoting plant health, aligning with the growing demand for sustainable agricultural practices.

## Conclusions

This study highlights the potential of *B. Subtilis* SK3- 24 biopellets as an effective, eco- friendly biocontrol strategy for managing tomato wilt caused by *R. solanacearum*. The biopellets demonstrated strong pathogen inhibition, comparable to chemical treatments, while ensuring prolonged bacterial viability and sustained release. In addition to disease suppression, the biopellets promoted tomato plant growth, likely through growth-promoting substances and nutrient solubilization. These results underscore the importance of integrating biocontrol agents into sustainable agricultural practices to reduce pesticide reliance. Future work should focus on field trials to validate efficacy under natural conditions, optimize biopellet formulations for enhanced stability, and explore the mechanisms of disease suppression and plant growth promotion. This research marks a key step toward

developing sustainable solutions for managing soilborne diseases and promoting agricultural productivity.

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Generative AI (ChatGPT, OpenAI) was used solely to improve the readability and language of the manuscript, under the full oversight of the authors. The authors remain entirely responsible for the content. AI tools were not listed as authors or co-authors.

**CRediT Author Statement**

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